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Repurposing the anti-viral drug zidovudine (AZT) in combination with meropenem as an effective treatment for infections with multi-drug resistant, carbapenemase-producing strains of *Klebsiella pneumoniae.*

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Abstract

Multi-drug resistant (MDR) *Klebsiella pneumoniae* represent a global threat to healthcare due to lack of effective treatments and high mortality rates. The aim of this research was to explore the potential of administering zidovudine (AZT) in combination with an existing antibiotic to treat resistant *K. pneumoniae* infections. Two MDR *K. pneumoniae* strains were employed, producing either the NDM-1 or KPC-3 carbapenemase. Efficacy of combinations of AZT with meropenem were compared with monotherapies against infections in *Galleria mellonella* larvae by measuring larval mortality and bacterial burden. The effect of the same combinations *in vitro* was determined via checkerboard and time-kill assays. *In vitro*, both *K. pneumoniae* strains were resistant to meropenem but were susceptible to AZT. In *G. mellonella*, treatment with either AZT or meropenem alone offered minimal therapeutic benefit against infections with either strain. In contrast,

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combination therapy of AZT with meropenem presented significantly enhanced efficacy compared to monotherapies. This was correlated with prevention of bacterial proliferation within the larvae but not elimination. Checkerboard assays showed that the interaction between AZT and meropenem was not synergistic but indifferent. In summary, combination therapy of AZT with meropenem represents a potential treatment for carbapenemase-producing MDR *K. pneumoniae* and merits further investigation.

Introduction

Klebsiella pneumoniae belongs to the family *Enterobacteriaceae* and is part of the normal intestinal flora of healthy humans. However, *K. pneumoniae* is also an important opportunistic pathogen and is a major cause of community and healthcare associated infections (HAIs) such as bloodstream, urinary tract infections and pneumonias (Navon-Venezia 2017). Infection is often associated with invasive medical devices or surgery in patients that are immunocompromised [reviewed in (Peleg and Hooper 2010)]. Compounding the problem, *K. pneumoniae* is adept at acquiring genes encoding a range of antibiotic resistance mechanisms via either mutations or mobile genetic elements [Navon-Venezia 2017]. Acquisition of resistance to third generation cephalosporins via extended-spectrum beta-lactamases (ESBLs) led to increased use of the antibiotics of 'last-resort' – the carbapenem class of broad-spectrum beta-lactams – resulting in the inevitable selection of resistance to these also. With increasing incidence of resistance to carbapenems, the prevalence of multi-drug, or extremely- drug resistant (MDR and XDR respectively), *K. pneumoniae* strains that are resistant to nearly all available antibiotics is rising globally (Sanchez 2013). Consequently, there are only a limited number of antibiotic treatments available (Morrill 2015) resulting in higher mortality rates (Munoz-Price 2013; Tamma 2017) and unsurprisingly, MDR or XDR *K. pneumoniae* are considered an urgent public health threat and a major challenge to the delivery of safe healthcare (World Health Organisation 2017).

The dearth of novel antibiotic treatments in the pipeline for Gram-negative bacteria means that the development of alternative treatments based on innovative use of existing drugs is an option. Clinically, the administration of antibiotic combinations to patients suffering from MDR or XDR *K. pneumoniae* infection is often employed as a solution to improve therapeutic outcomes. However, agreement on the best combinations to use, and evidence of any real benefit, is contested (Tamma 2012). Examples of combination therapies employed with some success include, dual carbapenem therapy (Souli 2017), ceftazidime with avibactam (Lagace-Wiens 2014), meropenem with vaborbactam (Lee and Baker 2018), and the polymyxin colistin combined with other antibiotics (Gutierrez-Gutierrez 2017). Unfortunately, none of these options are ideal particularly because of the nephrotoxicity and rise of resistance associated with colistin (Capone 2013), and the inability of either avibactam or vaborbactam to inhibit metallo-beta-lactamases such as NDM-1. Thus, there is an urgent clinical need to identify new treatment approaches for MDR or XDR *K. pneumoniae* strains that have acquired carbapenemases.

To improve options available for clinicians, the 'repurposing' of already approved drugs, whose primary use is not as antibacterials, as antibiotics could speed up the introduction of new treatment options, particularly if these compounds are administered in combinations with existing antibiotics (Cheng 2019; Ejim 2011). One example of a drug that could be 'repurposed' as an antibiotic is the anti-viral drug zidovudine (AZT). AZT is a nucleoside analogue with known bactericidal activity against Gram-negative bacteria, including *K. pneumoniae* (Elwell 1987; Lewin and Aymes 1989; Peyclit 2018). In addition, the drug was efficacious in a mouse model of systemic *Escherichia coli* infection (Keith 1989). Upon entering Gram-negative bacterial cells, AZT is phosphorylated by thymidine kinases, incorporated into DNA, and arrests replication by acting as a DNA chain terminator (Doleans-Jordheim 2011). A number of recent studies that screened libraries of approved drugs identified synergistic combinations against Gram-negative bacteria that included AZT (Hind 2019; Ng 2018; Wambaugh 2017). Synergistic combinations of AZT that inhibited MDR, or XDR, *K. pneumoniae in vitro* included combinations with the lipopeptide antibiotics colistin (Hu 2019) or polymyxin B (Lin 2019) that also showed enhanced efficacy compared to monotherapy in murine

infection models. Despite these studies with the lipopeptide antibiotics, most studies of the effect of AZT in combination with existing antibiotics have been performed *in vitro*.

The aim of this research was to explore further the potential of administering AZT in combinations with existing antibiotics to treat infections with MDR pathogens using the *Galleria mellonella* larvae infection model. This system permits screening of antibacterial activity *in vivo* against real infections, in the presence of a functioning immune system, without the high costs and ethical issues associated with mammalian infection models. Thus, combination treatments consisting of AZT with the carbapenem beta-lactam, meropenem, were screened for enhanced efficacy compared to monotherapies in *Galleria mellonella* larvae infected with carbapenemase-producing strains of *K. pneumoniae*.

Materials and Methods

Bacteria and growth media

K. pneumoniae strains were obtained from the National Collection of Type Cultures (NCTC; https://www.phe-culturecollections.org.uk/collections/nctc.aspx): NCTC 9633T, an antibiotic susceptible Type strain, NCTC 13443, producing the NDM-1 metallo-beta-lactamase, and NCTC 13438, producing the KPC-3 carbapenemase (Woodford 2008). NCTC 9633T was included as a control to illustrate the resistance of the two carbapenemase strains to meropenem. All strains were grown to stationary phase in Mueller–Hinton broth (MHB; Merck, Darmstadt, Germany) at 37^{o}C with shaking (at 200 rpm) overnight to prepare inocula for antibiotic efficacy testing *in vitro* or *in vivo*.

Drugs and *G. mellonella* larvae

Meropenem and zidovudine were purchased from Sigma–Aldrich Ltd (Dorset, UK). Stock solutions of meropenem or AZT were prepared in sterile deionized water with 10% dimethylsulphoxide. Sub-stocks of each drug for injection into larvae were prepared in deionized water. *G. mellonella* larvae were obtained from UK Waxworms Ltd (Sheffield, UK).

Antibiotic susceptibility testing *in vitro*

Minimum inhibitory concentrations (MICs) of antibiotics against the *K. pneumoniae* strains were determined in 96-well microplates as previously described (Hill 2014). Briefly, doubling dilutions of meropenem or zidovudine were prepared in MHB and subsequently inoculated with 1.0×10^6 cfu/mL of either *K. pneumoniae* strain. Microplates were incubated at 37°C and the MIC was defined as the concentration(s) present in the first optically clear well after 24 h.

Testing combinations for synergy *in vitro*

The effect of combinations of meropenem with AZT against both *K. pneumoniae* strains was carried out using 96-well microplate assays prepared via doubling dilution of meropenem in MHB followed by subsequent addition of AZT to form a combination checkerboard. Each well was then inoculated with 1.0×10^6 cfu/mL of either *K. pneumoniae* strain and microplates were incubated at 37ºC. After 24 h, each well was scored for visible growth and fractional inhibitory concentration index (FICI) values were calculated for each combination tested. Synergy was defined as FICI ≤0.5 (Eliopoulos 1996). Each *K. pneumoniae* strain was tested in duplicate.

The effect of meropenem and AZT combinations against both *K. pneumoniae* strains was also measured using a time-kill assay. Briefly, tubes containing concentrations of AZT, meropenem or a combination of both drugs were prepared in MHB broth. A control tube contained only sterile water in MHB. Drug concentrations were prepared at MIC_{50} for each strain. Tubes were then inoculated with 1.0×10^7 cfu/mL of either *K. pneumoniae* strain. Viability was determined after 0, 2, 4 and 6 h incubation at 37°C by serial dilution in MHB and plating on Nutrient Agar (Merck, Darmstadt, Germany). Each experiment was performed in duplicate and results expressed as mean ± standard error of the mean (SEM).

G. mellonella infection model

Efficacy of meropenem or AZT alone or in combination versus *G. mellonella* larvae infected with the *K. pneumoniae* strains was carried out exactly as described previously (Hill 2014; Krezdorn 2014;

Adamson 2015). *G. mellonella* at their final instar larval stage were kept at room temperature in darkness. Larvae weighing within the range of 250 to 350 mg were selected for each experiment to ensure consistency in subsequent drug administration and were used within 1 week of receipt. Groups of 15 larvae were infected with inocula (10 µL) of either *K. pneumoniae* strain containing increasing numbers of bacteria to determine an appropriate infectious dose for subsequent drug efficacy studies. Control experiments using a heat-killed inoculum of each strain was carried out in which the bacterial suspension was heated at 98ºC for 10 min prior to infection of a group of larvae. For all studies of drug efficacy, an inoculum of 5.6×10^5 cfu, or 9.1×10^7 cfu, was used for *K. pneumoniae* NCTC 13443 (NDM-1) and NCTC 13438 (KPC-3), respectively. A single treatment with phosphate-buffered saline (PBS), meropenem, AZT or a combination of both drugs was administered 2 h post-infection. The experiments were repeated in duplicate using larvae from a different batch and the data from these replicate experiments were pooled to give n=30. Survival data were plotted using the Kaplan–Meier method (Bland and Altman 1998) and comparisons made between groups using the log-rank test (Bland 2004). In all comparisons with the negative control it was the uninfected control (rather than the unmanipulated control) that was used. Holm's correction was applied to account for multiple comparisons in all tests and $p \leq 0.05$ was considered significant (Holm 1979).

Bacterial burden within larvae from each treatment group was measured exactly as described previously (Krezdorn 2014; Adamson 2015; Ballard and Coote 2016). Groups of 30 larvae were infected with either strain of *K. pneumoniae* using the same inoculum sizes as described above. Meropenem, AZT or a combination treatment of both drugs were administered at 2 h post-infection. Larvae were incubated in Petri dishes at 37°C. At 24 h intervals, five larvae were randomly selected from each treatment group and surface decontaminated and anaesthetised by washing in absolute ethanol. Each larva was then placed in an Eppendorf tube containing 1 mL of sterile PBS and homogenised using a sterile pestle. Bacterial burden from individual caterpillars was then determined by serial dilution of the homogenate in MHB and plating on MacConkey agar

(Formedium Ltd, Hunstanton, England). The detection limit for this assay was 100 cfu/mL of larval homogenate.

Results

Two carbapenemase-producing strains of *K. pneumoniae* are resistant to meropenem but sensitive to the antiviral drug AZT. In comparison to the antibiotic-susceptible Type strain, the two strains producing the carbapenemases displayed resistance to meropenem as expected (Table 1). The MICs of meropenem were the same for both carbapenemase-producing strains but *K. pneumoniae* NCTC 13438, carrying the KPC-3 carbapenemase, was more resistant to AZT (4 mg/L) than NCTC 13443 with NDM-1 (1 – 2 mg/L). These results confirmed previous studies with different strains of *K. pneumoniae* that showed sensitivity to AZT (Elwell 1987; Lewin and Aymes 1989; Peyclit 2018).

G. mellonella* larvae display dose-dependent lethality in response to infection by either strain of MDR *K. pneumoniae. The effect of infection with either *K. pneumoniae* strains on survival of *G. mellonella* is shown in Figure 1. In both strains, the heat-killed inoculum had no significant effect on larval survival ($p>0.05$). With live inocula, larval survival was affected in a dose-dependent manner during 96 h incubation (Figure 1). Together, these data indicate that infection with live *K. pneumoniae* is required to cause larval death and support previous studies that have utilised *G. mellonella* to study infection by different *K. pneumoniae* strains (Insua 2013; Wand 2013; McLaughlin 2014; Benthall 2015).

K. pneumoniae NCTC 13443 (NDM-1) displayed greater virulence than NCTC 13438 (KPC-3), requiring a smaller inoculum of viable bacteria to induce a similar degree of lethality to infected larvae. Infectious doses for each strain were selected for use in subsequent studies on antibiotic efficacy (5.6×10^5 cfu for *K. pneumoniae* NCTC 13443, and 9.1×10^7 cfu for NCTC 13438) that resulted in the death of approximately 90% of larvae after 96 h incubation at 37ºC.

Efficacy of AZT or meropenem monotherapy in *G. mellonella* larvae infected with either carbapenemase-producing strain of *K. pneumoniae* is poor. The effect of single doses of AZT or meropenem, 2 h post-infection (p.i) with either *K. pneumoniae* strain, on survival of *G. mellonella* larvae is shown in Figure 2. Both drugs increased larval survival in a dose-dependent manner regardless of the *K. pneumoniae* strain. However, neither drug induced high levels of therapeutic benefit, with the exception of AZT versus larvae infected with *K. pneumoniae* NCTC 13443 (NDM-1) treated with the highest doses of 6.25 or 12.5 mg/kg. In contrast, treatment of larvae infected with the KPC-3-producing strain with the same doses of AZT had no therapeutic benefit after 120 h. As would be expected with these strains, treatment with meropenem induced only low levels of therapeutic benefit even at the highest doses tested, particularly against infections with *K. pneumoniae* NCTC 13438 (KPC-3).

In summary, neither AZT or meropenem monotherapy offered high levels of therapeutic benefit versus infections with either strain of carbapenemase-producing *K. pneumoniae*, although both drugs were moderately more effective against the NMD-1-producing strain than the KPC-3 strain.

Combination therapy with AZT plus meropenem shows enhanced efficacy compared to monotherapy versus *G. mellonella* infections with carbapenemase-producing MDR strains of *K. pneumoniae*. The effect of monotherapy, with AZT or meropenem, compared with combination therapy on survival of *G. mellonella* larvae, and burden of infecting bacteria, for both carbapenemase-producing strains of *K. pneumoniae* is shown in Figure 3. Pilot experiments were carried out testing many different doses of AZT or meropenem in combination to identify the most effective doses to employ against larval infections with either strain of *K. pneumoniae* (data not shown). From these initial studies, optimal combination dosing regimens were selected that offered the best therapeutic benefit and subsequently studied in detail – NCTC 13443 (AZT - 0.78 mk/kg + meropenem - 6.25 mg/kg) and NCTC 13438 (AZT - 6.25 mg/kg + meropenem - 12.5 mg/kg) (Figure 3).

As shown previously (Figure 2), a single dose 2 h p.i of either AZT or meropenem had minimal therapeutic benefit on larvae infected with either strain of *K. pneumoniae* (Figure 3). This was

reflected in large increases over 24 h in the internal burden of either infecting *K. pneumoniae* strain within individual larvae. For larvae infected with NCTC 13443 (NDM-1), the increase in bacterial numbers after treatment with either meropenem or AZT was the same as larvae mock treated with PBS. With strain NCTC 13438 (KPC-3), larvae treated with either meropenem or PBS alone also displayed an identical increase in bacterial burden after 24 h. Notably, the increase in bacterial numbers after treatment with AZT was reduced (approximately 0.5 $\log_{10}$ cfu/mL), indicating some inhibitory effect of the drug on proliferation of strain NCTC 13438 within the larvae. However, this smaller increase in bacterial numbers post AZT therapy was not reflected in any significant reduction in larval death compared to treatment with meropenem or PBS (Figure 3).

In direct contrast to monotherapies, a single dose of a combination of AZT + meropenem resulted in significantly ($p \leq 0.05$) enhanced survival of larvae infected with either MDR strain of *K. pneumoniae* (Figure 3). For example, 120 h p.i with NCTC 13443 (NDM-1), populations treated with PBS, AZT alone or meropenem alone showed survival of 20, 40 and 47% respectively. In contrast, 67% of larvae treated with the combination survived. At the same time p.i with NCTC 13438 (KPC-3), treatment with PBS, AZT alone or meropenem alone showed survival of 10, 13 and 17% respectively compared to 47% for the combination. Correlating with this enhanced survival, the burden of bacteria within the infected larvae did not show the large increase after 24 h observed previously with the monotherapies, and, bacterial numbers remained significantly lower throughout the duration of the experiment ($p \leq 0.05$). Whilst the combination treatment halted the proliferation of infecting bacteria of either strain, numbers did not fall below the initial infecting inoculum size. Thus, the therapeutic benefit conferred by AZT+ meropenem combination treatment appears to be due to a bacteriostatic effect *in vivo*.

In summary, a combination treatment of AZT + meropenem offers a potential novel therapy for treatment of MDR, carbapenemase-producing strains of *K. pneumoniae*.

The inhibitory action of the combination of AZT plus meropenem versus *K. pneumoniae* is not significantly synergistic *in vitro*. To help understand the nature of the inhibitory action of the combination of AZT with meropenem that conferred enhanced efficacy *in vivo*, checkerboard and

time-kill experiments were conducted *in vitro* (Figure 4). A checkerboard assay showing the effect of different AZT and meropenem combinations on growth of both carbapenemase-producing strains of *K. pneumoniae* is shown in Figure 4a. For NCTC 13443 (NDM-1), there was some evidence of minor synergy (FICI ≤ 0.5) at only two combinations tested. The majority of the other combinations that inhibited growth did so in an indifferent or additive fashion. For strain NCTC 13438 (KPC-3), none of the inhibitory combinations tested were synergistic, with all displaying indifference or additivity.

The effect of exposure to the single drugs (at MIC_{50}) and a combination (also at MIC_{50} for each drug) on viability of both strains is shown in Figure 4b. Bacterial viability was measured over a period of 6 h at 37°C. Control populations of both strains, mock treated with PBS increased in cell number over the duration of the experiment. Exposure to AZT alone resulted in a loss of viability of both strains of approximately 3 $\log_{10}$ cfu/mL after 6 h. In contrast, exposure to meropenem also resulted in loss of viability, but after 6 h exposure, there was evidence from both strains that the surviving population of bacteria was recovering and growth resuming. With both strains, the combination treatment resulted in a steady decline in viability of approximately 4 $\log_{10}$ cfu/mL after 6 h. Despite the greater bactericidal effect of the combination compared to the individual drugs, the loss of viability induced by the combination was only approximately 1 $\log_{10}$ cfu/mL more than AZT alone. This supports the checkerboard results by showing that the inhibition of *K. pneumoniae* induced by exposure to the combination of AZT with meropenem is not strongly synergistic. Notably, despite being bactericidal, the combination did not eliminate all bacteria over the duration of the experiment. This observation is supported by the *G. mellonella* larval burden assays (Figure 3) where infecting *K. pneumoniae* were never eliminated and a reduced number of bacteria were always detectable.

Discussion

The antibacterial properties of AZT have been well documented but the drug has never been formally approved to treat bacterial infections (Elwell 1987; Lewin and Aymes 1989; Ng 2018). With the emergence of untreatable infections by MDR, or XDR, Gram-negative bacteria, there has been renewed interest in exploiting these properties and ‘repurposing’ the drug. In fact, a recent study

proposed using AZT alone as a salvage therapy for colistin-resistant infections (Peyclit 2018). In this study, AZT was shown to inhibit two MDR, carbapenemase-producing strains of *K. pneumoniae* with MICs between 1.0 and 4.0 mg/L, confirming the known inhibitory effect of AZT on this pathogen (Elwell 1987; Lewin and Aymes 1989; Peyclit 2018). In addition, *G. mellonella* larvae infected with the same strains displayed enhanced survival after monotherapy with AZT.

Despite many studies showing that AZT is antibacterial, one reason why the drug may not be highly effective as a monotherapy is the induction of resistance after short-term exposure (Lewin 1990). AZT only inhibits bacteria that possess a thymidine kinase that phosphorylates the AZT such that it can then be incorporated into DNA and arrest DNA replication (Doleans-Jordheim 2011). In *E. coli*, mutations, or the presence of insertion sequences, in the gene encoding thymidine kinase (that could result in impaired function of the enzyme) correlated with AZT resistance (Doleans-Jordheim 2011). Furthermore, *E. coli* strains resistant to AZT have been isolated from patients undergoing therapy with the drug and the activity of thymidine kinase in these strains was reduced (Lewin 1990). Thus, because of the issue of resistance to AZT, the most likely application of the drug as an antibacterial therapy for MDR Enterobacteriaceae is in combination therapies with antibiotics that could help reduce the onset of resistance.

A number of studies have highlighted effective combinations of AZT with various approved antibiotics i*n vitro* including: tigecycline against MDR *E. coli* and *K. pneumoniae* (Ng 2018); colistin versus colistin-resistant *K. pneumoniae* (Falagas 2019) or *E. coli* (Loose 2018; Peyclit 2018); trimethoprim and/or sulfamethizole against trimethoprim-resistant *E. coli* and *K. pneumoniae* clinical isolates (Wambaugh 2017); and the aminoglycosides, gentamicin and amikacin against *E. coli* (Doleans-Jordheim 2011). Notably, two studies demonstrate enhanced efficacy *in vivo* of AZT in combination with colistin (Hu 2019) or polymyxin B (Lin 2019), compared to their constituent monotherapies, in murine infection models with NDM-producing *K. pneumoniae* and/or colistin-resistant *E. coli*. In this study, a combination therapy consisting of AZT with meropenem resulted in enhanced efficacy against infections by two MDR, carbapenemase-producing strains of *K. pneumoniae* in *G. mellonella* larvae compared to each monotherapy. Supporting these findings, a

recent screen of Food and Drug Administration-approved drugs identified that AZT acted as an antibiotic-resistance breaker (ARB) when combined with meropenem and potentiated the inhibitory effect of the antibiotic against MDR *K. pneumoniae in vitro* (Hind 2019). Clearly, only two NCTC carbapenemase-producing strains were used in this study and additional studies using a range of carbapenemase-producing clinical isolates will be required to confirm the enhanced efficacy of this combination.

Despite the enhanced efficacy of the combination, the larval populations treated with the combinations still suffered mortality for the duration of the experiments albeit less than those treated with the monotherapies. This observation could be explained by the effect the different treatments had on the burden of infecting bacteria within the larvae. For example, monotherapies had no detrimental effect on the infecting bacteria of either strain because numbers increased rapidly over the first 24 h in the same fashion as larvae sham-treated with PBS. However, combination therapy had the effect of preventing infecting bacteria of either strain from proliferating in the larvae but, notably, did not reduce bacterial burden. The fact that combination-treated larvae still contained viable bacteria could account for these populations suffering mortality at a reduced rate compared to populations treated with either monotherapy. These observations *in vivo* were supported by the results from the *in vitro* time-kill experiments. For example, despite the combination of AZT with meropenem showing a bactericidal effect, lethality slowed over a 6 h period of exposure and low numbers of either strain survived. Furthermore, the enhanced efficacy of the combination treatment *in vivo* was unlikely to be due to a synergistic interaction between AZT and meropenem because the *in vitro* experiments largely showed an indifferent or additive effect. The lack of potent synergy between AZT and meropenem versus the two *K. pneumoniae* strains could account for the observed survival kinetics of infected larvae and the failure of the combination treatment to confer full survival or eliminate all infecting bacteria at the doses tested. It is likely that the enhanced efficacy of the combination observed *in vivo* can be explained by the FICI values observed *in vitro*. For example, the best FICI value obtained for the NDM-1 strain was 0.5 and for the KPC-3 strain 0.62. These values represent a weak synergistic effect or, at the very least, an indifferent or additive

effect of the combination versus both strains. An additive effect is supported by the time-kill assay whereby a 6 h exposure to the combination resulted in only approximately 1 $\log_{10}$ cfu/mL greater reduction in cell numbers than either of the constituent drugs alone.

For therapy of Human Immunodeficiency Virus (HIV), AZT is administered orally at 300 mg twice a day indicating that blood plasma concentrations above the MIC for *K. pneumoniae* could be reached (Peyclit 2018; Falagas 2019). Furthermore, AZT is well-tolerated, and toxicity generally only manifests after long-term use of the drug – a scenario that would be unlikely if antibiotic/AZT combinations were used to treat acute bacterial infections.

In summary, this work has identified that combination of AZT with meropenem represents a plausible alternative therapy to treat infections with MDR, carbapenemase-producing strains of *K. pneumoniae* and merits further investigation.

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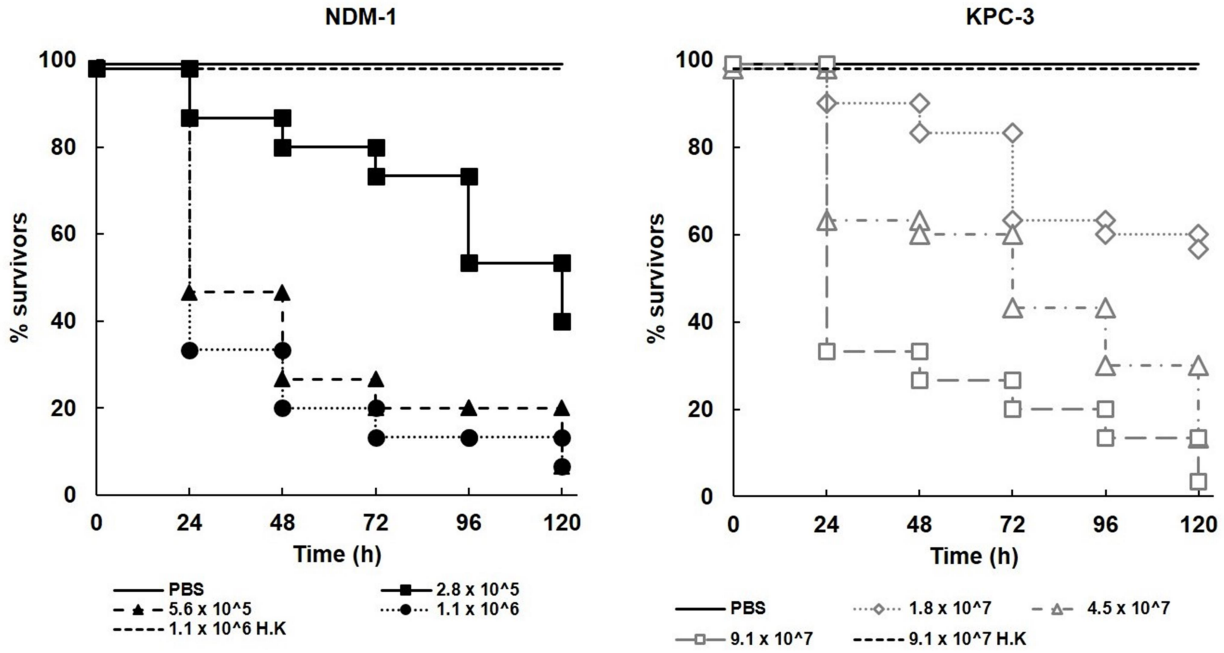

Figure 1. Effect of increasing inoculum dose of live *K. pneumoniae* NCTC 13443 (NDM-1) or NCTC 13438 (KPC-3) on the survival of *G. mellonella* larvae during incubation at 37°C for 120 h. Numbers in the legend indicate the inoculum size in bacterial cells per larva. For both strains, the effect of heat-killed (h.k.) bacterial inocula is also shown. No significant mortality was observed in an unmanipulated group (data not shown) or in the uninfected group mock 'infected' with sterile PBS. For all infected groups, survival was significantly reduced compared to the mock 'infected' group ($p<0.05$, log rank test with Holm correction for multiple comparisons); n=30 (pooled from replicate experiments).

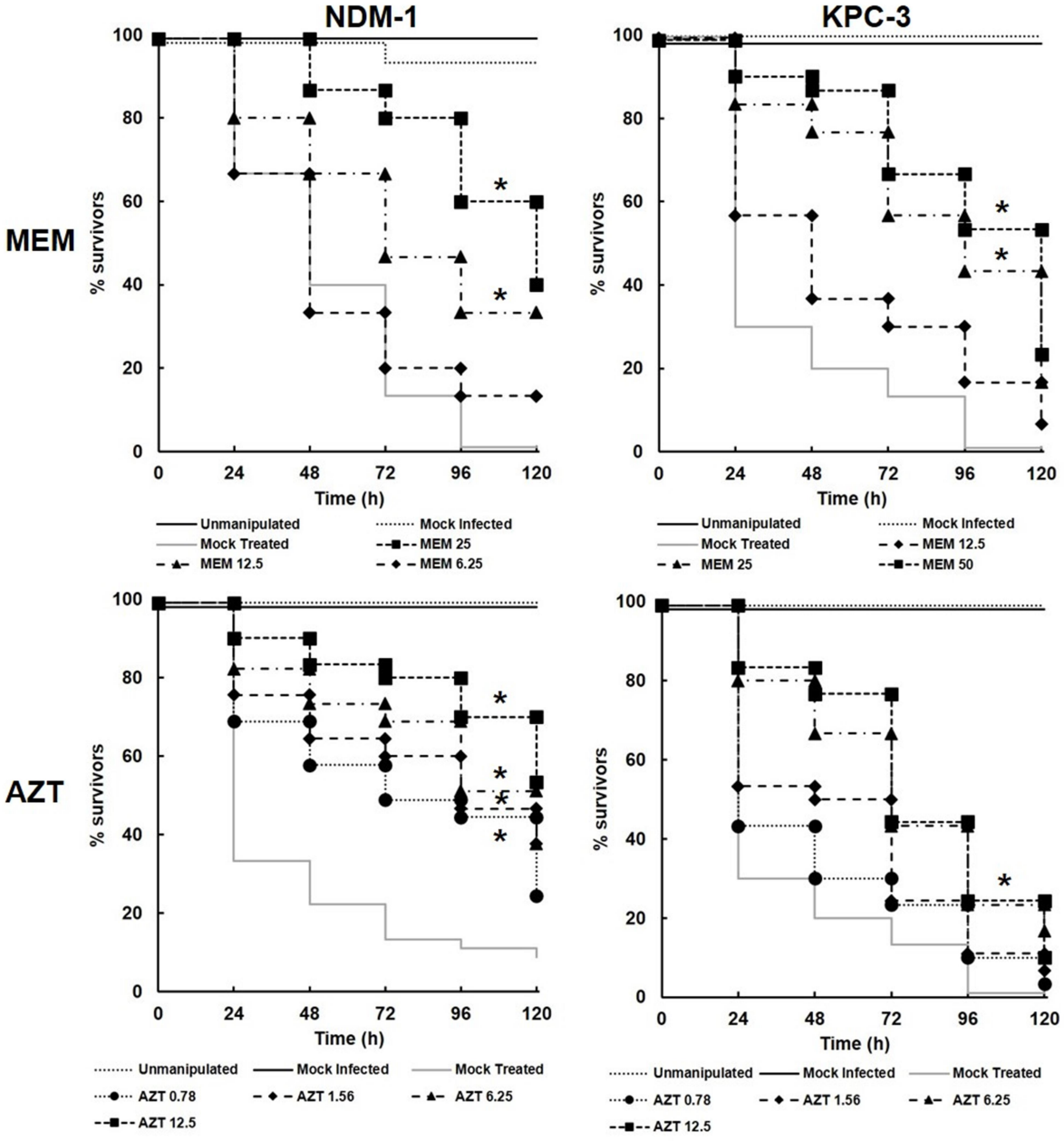

Figure 2. Effect of treatment with zidovudine (AZT) or meropenem (MEM) on survival of *G. mellonella* larvae infected with *K. pneumoniae* strains. Groups of larvae were mock 'infected' with sterile PBS, or 5.6 x 10^5 cells of the NDM-1 strain, or 9.1 x 10^7 cells of the KPC-3 strain. 2 h post-infection (p.i), a single dose of either MEM or AZT was administered (dose in mg/kg is the number shown on the figure). The mock 'treated' group represents infected larvae treated with sterile PBS.

Larval survival was measured over a period of 120 h at 37°C. * indicates significantly enhanced survival compared to the mock ‘treated’ group (p<0.05, log rank test with Holm correction for multiple comparisons); n=30 (pooled from duplicate experiments).

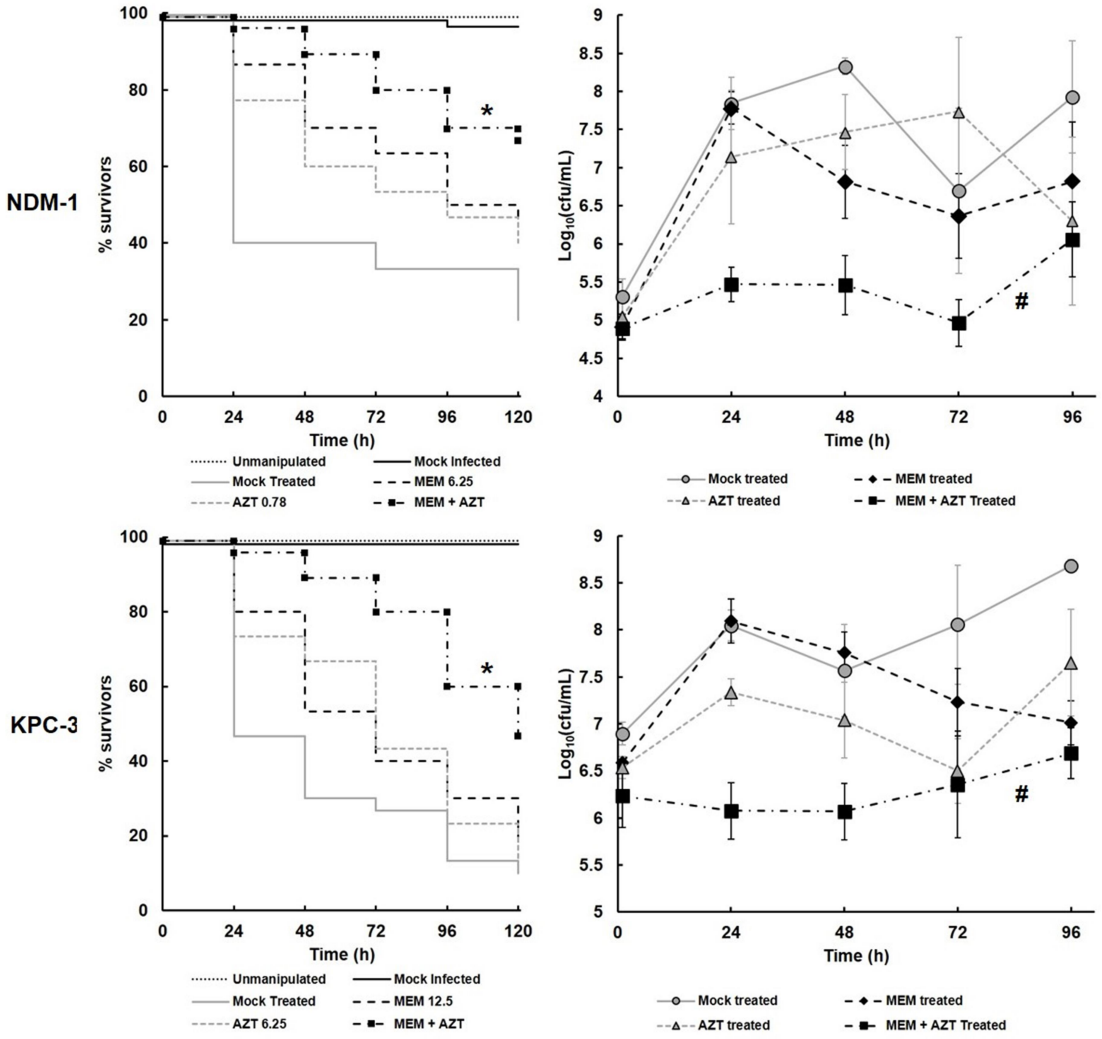

Figure 3. Effect of treatment with combinations of AZT and MEM on the survival and internal bacterial burden of *G. mellonella* larvae infected with *K. pneumoniae* strains. Larvae were infected with PBS (mock 'infected'), *K. pneumoniae* NCTC 13443 (NDM-1) – 5.6 x 10^5 cells, or NCTC 13438 (KPC-3) – 9.1 x 10^7 cells and treated with either PBS (mock 'treated'), or a single dose of each drug individually, or a combination of MEM and AZT at 2 h p.i. (dose in mg/kg is the number shown on the figure). Larvae were incubated at 37°C for 120 h and survival recorded every 24 h.

The larval burden of *K. pneumoniae* was determined from five individual larvae per treatment group every 24 h for 96 h at 37°C. Error bars indicate ±SEM.

* combination treatment group with significantly enhanced survival compared with any of the constituent monotherapies ($p < 0.05$, log-rank test with Holm's correction for multiple comparisons). n=30 (pooled from duplicate experiments).

significant difference in larval burden between groups treated with the combination of AZT + MEM compared with each monotherapy ($p < 0.05$, the Mann–Whitney U-test compared the combination therapy with each monotherapy). $n = 5$.

NDM-1

AZT (mg/L)	0.25	0.5	1	2	4	8	16	32	64	128	256
2	1.0	1.0	1.0	1.01	1.01	1.03	1.06	1.12	1.25	1.5	2
1	0.5	0.5	0.5	0.51	0.51	0.53	0.56	0.62	0.75	1.0	1.5
0.5	0.25	0.25	0.25	0.26	0.26	0.28	0.31	0.37	0.5	0.75	1.25
0.25	0.13	0.13	0.13	0.13	0.14	0.15	0.19	0.25	0.37	0.62	1.12
0.125	0.06	0.06	0.07	0.07	0.08	0.09	0.12	0.19	0.31	0.56	1.06
0.062	0.03	0.03	0.03	0.04	0.05	0.06	0.09	0.15	0.28	0.53	1.03
0.031	0.02	0.02	0.02	0.02	0.03	0.05	0.08	0.14	0.26	0.51	1.01

Meropenem (mg/L)

KPC-3

AZT (mg/L)	0.25	0.5	1	2	4	8	16	32	64	128	256
4	1.0	1.0	1.01	1.02	1.03	1.06	1.12	1.25	1.5	2.0	3.0
2	0.5	0.5	0.51	0.52	0.53	0.56	0.62	0.75	1.0	1.5	2.5
1	0.25	0.25	0.26	0.26	0.28	0.31	0.37	0.5	0.75	1.25	2.25
0.5	0.13	0.13	0.13	0.14	0.16	0.19	0.25	0.37	0.62	1.12	2.12
0.25	0.06	0.07	0.07	0.08	0.09	0.12	0.19	0.31	0.56	1.06	2.06
0.125	0.03	0.03	0.04	0.05	0.06	0.09	0.15	0.28	0.53	1.03	2.03
0.062	0.02	0.02	0.02	0.03	0.05	0.08	0.14	0.27	0.52	1.02	2.02

Meropenem (mg/L)

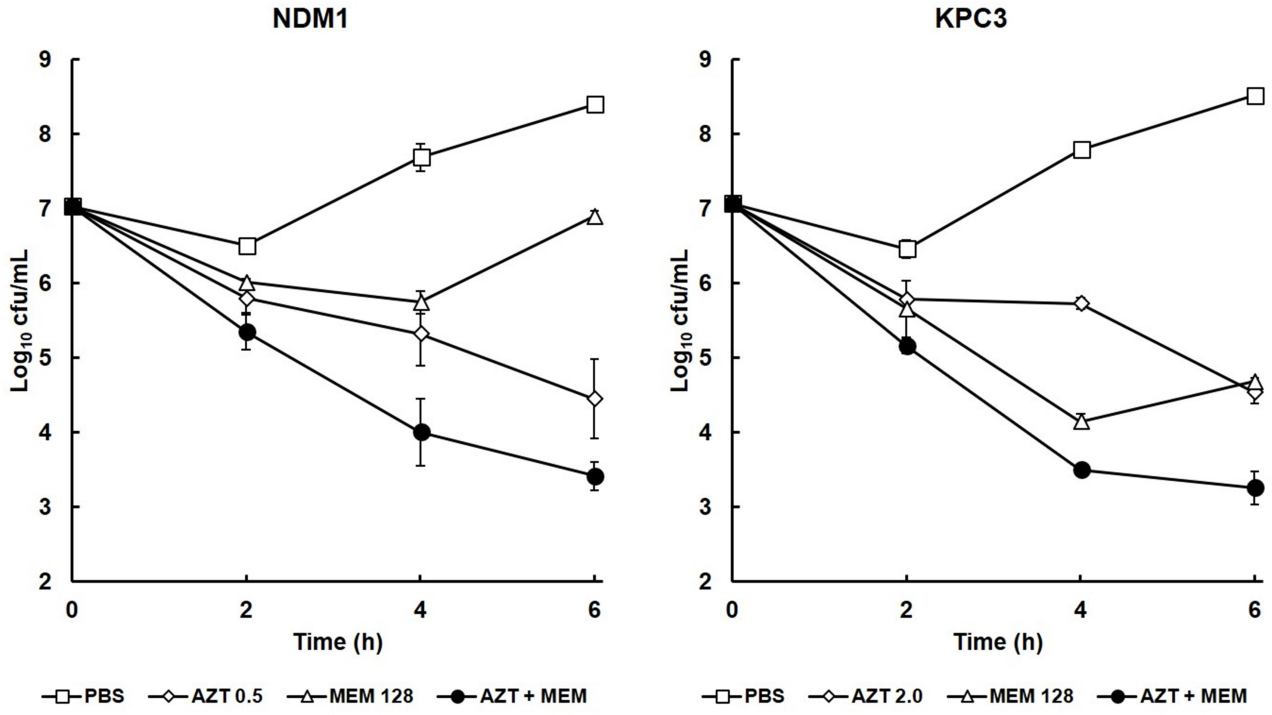

Figure 4. The effect of combination of AZT with MEM on the growth and viability of *K. pneumoniae* NCTC 13443 (NDM-1) or NCTC 13438 (KPC-3) *in vitro*. Fractional inhibitory concentration indices (FICI) of AZT combined with MEM versus NDM-1 and KPC-3 after 24 h in MHB at 37^{o}C (A). Black squares indicate FICI values where bacterial growth occurred. Grey squares indicate wells where the FICI values were ≥ 0.5 (indicating inhibition was not synergistic). White squares show FICI values of 0.5 or less where bacterial growth was inhibited and thus indicate synergistic inhibition of growth. The experiment was performed in duplicate and a representative result is shown.

Time-kill curves of the effect of 6 h exposure to PBS or MIC_{50} of; MEM alone (128 mg/L for both NDM-1 and KPC-3); AZT alone (0.5 mg/L NDM-1 and 2 mg/L KPC-3) or AZT + MEM (NDM-1 – 128 mg/L MEM + 0.5 mg/L AZT; KPC-3 – 128mg/L MEM + 2 mg/L AZT). Error bars indicate ±SEM from duplicate experiments (B).

Table 1 – Minimum inhibitory concentration (MIC) of zidovudine (AZT) and meropenem (MEM) for the *K. pneumoniae* Type strain or strains possessing either the NDM-1 or KPC-3 carbapenemases.

Bacterial Strains	**MIC (mg/L)**	
	AZT	**MEM**
***K. pneumoniae* NCTC 9633T**	-	<0.0625
***K. pneumoniae* NCTC 13443 (NDM-1)**	1.0 – 2.0	128 - 256
***K. pneumoniae* NCTC 13438 (KPC-3)**	4.0	128 - 256